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(71) Applicant: AMERSHAM PHARMACIA BIOTECH INC. [US/US]; 800 Centennial Avenue, Piscataway, NJ 08855-1327 (US).

(72) Inventors: RIEDL, William, A.; 378 Vista View Drive, Cedarburg, WI 53012 (US). FLY, Susan, J.; 1300 Greenway Terrace #1, Brookfield, WI 53005 (US). KABOORD, Barbara, F.; 3719 West Haven Avenue, Mequon, WI 53092 (US). NYE, Steven, H.; 10404 North Riverview Court, Mequon, WI 53092 (US). TING, Eve, Y.; 230 Bunker Hill Drive, Brookfield, WI 53005 (US).

(74) Agent: BAKER, Jean, C.; Quarles & Brady, 411 East Wisconsin Avenue, Milwaukee, WI 53202-4497 (US).

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(54) Title: *THERMOCOCCUS BAROSII* DNA POLYMERASE MUTANTS

(57) Abstract

A *Thermococcus barosii* DNA polymerase with reduced 3'-5' exonuclease activity is disclosed. Additionally disclosed is a *Thermococcus barosii* DNA polymerase with increased ability to incorporate ribonucleotides and dideoxynucleotides.

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THERMOCOCCUS BAROSII DNA POLYMERASE MUTANTS

CROSS-REFERENCE TO RELATED APPLICATION

Not applicable.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

Thermococcus barosii is a thermophilic organism obtained from deep vent flange, Endeavor Segment, Juan de Fuca Ridge, off the coast of Washington State in the U.S.A. Native *Tba* (*nTba*) DNA polymerase has been purified from cell paste by classical chromatographic purification methods including Q-Sepharose, Heparin Sepharose, phosphocellulose and Mono-Q (U.S. patent 5,602,011). Characterization of the purified *nTba* showed that it possesses an active proofreading function in addition to its DNA-dependent DNA polymerase activity. *Tba* DNA polymerase does not possess detectable 5'-3' exonuclease activity. Because *nTba* DNA polymerase was obtained from a thermophilic organism, it was shown to retain polymerase activity following prolonged treatment at elevated temperatures (95°C).

U.S. patent 5,602,011 discloses cloned *Tba* DNA polymerase.

Cloning of the gene for the *Tba* DNA polymerase DNA polymerase revealed that it is a member of the alpha-family of DNA polymerases and is approximately 80%

conserved compared with the Pfu and Deep Vent DNA polymerases.

Based on amino acid sequence comparisons and protein expression studies on the truncated forms of other known polymerases, it can be shown that the organization of the gene is structured such that the 3'-5' exonuclease function is encoded in the 5' half of the gene, while the polymerase function is encoded in the latter half. The predicted amino acid sequence derived from the gene sequence highlights a metal binding site in the 3'-5' exonuclease domain. This domain is typified by the amino acid sequence FDIET and is conserved between *Tba* DNA polymerase, Pfu and Deep Vent. (See, for example, Vemori, et al., Nucl. Acids Research 21(2):259-265, 1993.)

Alteration of the FDIET sequence to FAIAT has been shown in some polymerases to eliminate the proofreading function. (Derbyshire, V., et al., Science 240:199-201, 1988; Bernad, A., et al., Cell 59:219-228, 1989; Frey, M.W., et al., Proc. Natl. Acad. Sci. USA 90:2579-2583, 1993; Mather, E.J., U.S. patent 5,489,523.)

BRIEF SUMMARY OF THE INVENTION

In one embodiment, the present invention is a *Thermococcus barosii* DNA polymerase with a reduced 3'-5' exonuclease activity. Preferably, the exonuclease activity is reduced at least 50% as measured by the ability of the polymerase to incorporate dNTPs into an

oligonucleotide primer with mismatches. In a most preferable form of the invention, the reduction is at least 75% or 90%.

In one preferred form of the invention, the FDIET amino acid sequence (residues 140-144 of native *Tba* DNA polymerase) has been altered to FAIAT.

In another embodiment, the present invention is a polymerase with a reduced 3'-5' exonuclease activity wherein amino acid residue 489 has additionally been altered. In a preferred form of the present invention, residue 489 has been altered to either a tyrosine or a phenyalanine. In a most preferred form of the present invention, the residue is a tyrosine. In this embodiment, the polymerase has an increased ability to incorporate dideoxynucleotides and ribonucleotides compared to a *Tba* DNA polymerase with no alteration at residue 489. The ability to incorporate dideoxynucleotides is preferably at least 2-fold higher, and most preferably at least 3-fold higher than the unaltered *Tba* DNA polymerase. The ability to incorporate ribonucleotides is preferably at least 2-fold higher, more preferably at least 3-fold higher, and most preferably at least 5-fold higher than unaltered *Tba* DNA polymerase.

It is an object of the present invention to provide a DNA polymerase with increased utility for specific DNA polymerization applications that require a DNA polymerase with reduced exonuclease activity and increased ability

to incorporate dideoxynucleotides and ribonucleotides. Most preferably, this DNA polymerization method is a method of arrayed primer extension.

It is another object of the present invention to provide a DNA polymerase with an altered residue 489.

Other objects, advantages, and features of the present invention will become apparent after one has reviewed the specification and claims.

DETAILED DESCRIPTION OF THE INVENTION

In one embodiment, the present invention is a *Thermococcus barosii* DNA polymerase with reduced 3'-5' exonuclease activity. In a preferred form of the polymerase, the polymerase also has an altered ability to incorporate dideoxy and ribonucleotides into a polymerization product.

By "*Tba* DNA polymerase" is meant a DNA polymerase corresponding to one naturally isolated from *Thermococcus barosii*. SEQ ID NO:4 is an amino acid sequence of one preferred form of *Tba* DNA polymerase. SEQ ID NO:5 is a nucleic acid sequence of a preferred *Tba* DNA polymerase.

The Examples below described one preferred method for obtaining the *Tba* DNA polymerase of the present invention. Briefly, the inventors have obtained a recombinant clone of *Tba* DNA polymerase. A recombinant clone can be obtained from the *T. barosii* strain deposited at DSM on November 1, 1994 at Accession No. 9535. One would use standard methods of molecular

biology to produce probes from SEQ ID NOs:4 and 5 capable of screening a genomic DNA preparation created from DSM 9535 or other *Tba* strain. These techniques could be used to create a recombinant clone suitable for producing a polymerase of the present invention. U.S. patent 5,602,011, hereby incorporated by reference, describes a preferred method of obtaining a polymerase clone.

The inventors used standard methods of site-directed mutagenesis to replace the amino acid residues FDIET (SEQ ID NO:1) with FAIAT (SEQ ID NO:2). (SEQ ID NO:1 occurs at residues 140-144 of the native *Tba* DNA polymerase sequence.) This particular alteration renders the polymerase deficient in 3'-5' exonuclease activity.

The Examples also disclose the alteration of residue 489 and the subsequent creation of a *Tba* DNA polymerase with altered ability to incorporate dideoxy and ribonucleotides. In the Examples below, the residue 489 was substituted with a tyrosine residue. We envision that a phenylalanine residue would be equally successful. We also envision that other amino acids could be substituted at residue 489 to create an equally successful polymerase. We envision that certain substitutions will provide a polymerase with greater ability to incorporate dideoxynucleotides and ribonucleotides than those described below.

A preferable DNA polymerase of the present invention has a reduced 3'-5' exonuclease activity that is reduced at least 50% as measured by the ability of the polymerase

to incorporate dNTPs into an oligonucleotide primer with mismatches. The Examples below demonstrate this procedure. Preferably, the reduction is at least 75%. More preferably, the reduction is 90%.

In another embodiment, the DNA polymerase of the present invention has an increased ability to incorporate dideoxynucleotides as compared to *Tba* DNA polymerase with no alteration at residue 489. Preferably, this increase is at least 2-fold. More preferably, the increase is at least 3-fold. The increase can be measured by methods described below in the Examples.

In another embodiment, the *Tba* DNA polymerase of the present invention has an increased ability to incorporate ribonucleotides compared to *Tba* DNA polymerase with no substitute at residue 489. Preferably, the increase is at least 2-fold. More preferably, the increase is at least 3-fold and most preferably the increase is at least 5-fold.

In another embodiment, the present invention is a method of DNA polymerization comprising exposing the polymerase as described above to a DNA template, primer, dNTPs, and other reagents necessary for DNA polymerization. One particularly relevant DNA polymerization diagnostic application may be for an emerging "chip" technology referred to as Arrayed Primer Extension (APEX). In this method, DNA polymerases incorporate dye-terminators (eg. FL-ddNTPs) into bound

oligonucleotide primers that are arrayed onto a solid support.

In one example, APEX technology is used to determine potential mutations in human DNA by amplifying a human target template gene sequence and exposing the sequence via APEX to a primer array. Upon successful hybridization and primer extension, the sequence of the template can be determined and potential mutations detected.

A *Tba* DNA polymerase mutant that prefers ddNTPs is useful in APEX for two reasons. First, template DNA samples may be generated by PCR from genomic DNA of human patients. Because the template preparation may be contaminated with free deoxynucleotides carried into the chip reaction, the deoxynucleotides may effectively compete away incorporation of the dideoxy terminators. A *Tba* DNA polymerase mutant may be isolated that prefers ddNTPs and also discriminates against dNTPs. This mutant polymerase would thereby eliminate this problem. Second, the *Tba* DNA polymerase mutant that better incorporates ddNTPs relative to the wild-type *Tba* DNA polymerase may enhance the overall sensitivity of the APEX reaction on the chip. The combination of these effects may be crucial to the overall sensitivity of the technique and thus drive success of using a primer extension reaction in chip-based technology.

EXAMPLES

A. *Tba*(exo-) DNA polymerase

Methods: Sequencing and site-directed modification of the *rTba* gene

The sequence of a recombinant clone of *Tba* DNA polymerase in pUC18 vector (called pUC18, *rTba*) was verified on both strands by automated DNA sequence analysis. (pUC18, rTba is described in U.S. patent 5,602,011) CY5-labeled oligonucleotide primers that would hybridize throughout the presumed *Tba* DNA polymerase coding sequence were synthesized and purified utilizing C18 syringe cartridges. Dideoxy-sequencing reactions with T7 DNA polymerase were carried out in microwell dishes with approximately 5-10 μg of purified pUC18, *rTba* plasmid DNA according to manufacturer's specifications (AutoRead Sequencing Kit, Pharmacia Biotech, Uppsala, Sweden) and electrophoresed using the ALFexpress automated DNA sequencing apparatus (Pharmacia Biotech). DNA sequence comparisons were made using DNASTAR alignment software (DNASTAR, Inc., Madison, WI).

The recombinant *Tba* DNA polymerase coding region was cloned into the pET22b vector (Novagen, Madison, WI) to enable expression in bacteria to be driven from the bacteriophage T7 promoter. The 2.1 kb NdeI-XmaI and 275 bp XmaI-EcoRI fragments that span the full-length *rTba* coding region were isolated from pUC18, *rTba* and ligated into pET22b that was previously digested with NdeI and EcoRI and then dephosphorylated with calf intestinal

phosphatase (Pharmacia Biotech). The correct construct was isolated by restriction endonuclease mapping and the sequence at the cloning junctions verified by automated DNA sequence analysis.

DNA resequencing identified a one bp deletion in the *Tba* DNA polymerase coding region that deviated from the sequence originally reported in U.S. patent 5,602,011. A deletion of "A" occurs normally where GAG would encode glutamic acid at amino acid residue number 666 and results in the stop codon TAA to be aligned in-frame two codon positions downstream of the defect. To repair the defective gene, an oligonucleotide primer (repair primer 1) was synthesized that would hybridize to the sense region of interest and enable insertion of "A" in one round of PCR when used in conjunction with an antisense vector oligonucleotide. Another oligonucleotide (repair primer 2) was synthesized that includes a 5' BlpI restriction endonuclease site to extend the round one product and enable a BlpI-BlpI replacement fragment to be inserted into the *Tba* DNA polymerase coding region. The restored sequence encodes a full-length recombinant *Tba* DNA polymerase in pET22b (termed p22b, *rTba*FL).

A *rTba*(exo-) expression vector was constructed using overlapping PCR mutagenesis to introduce alanines (A) in place of aspartic (D) and glutamic (E) acid in a metal binding site (FDIET, SEQ ID NO:1) located within the presumed 3'-5' exonuclease domain. "FDIET" is located at residues 140-144. Two mutagenic primers encoding *E.*

coli-preferred codons were used in round one of PCR to generate DNA fragments that overlap at the corresponding FAIAT, SEQ ID NO:2, sequence. (These primers are listed at SEQ ID NOs:6 - 9. SEQ ID NOs:6 and 7 are the 5' set. SEQ ID NOs: 8 and 9 are the 3' set.) A mixture of these fragments in a PCR reaction, with a sense primer located 5' to the SacII site and an antisense primer positioned 3' to the BamHI site, amplified a *Tba* DNA polymerase fragment containing FAIAT in place of FDIET. Following restriction endonuclease digestion, the SacII-BamHI mutagenic fragment was cloned in place of the normal *Tba* DNA polymerase sequence to create vector p22b, *rTba*(exo$^-$).

All large scale plasmid DNA preparations were obtained from 350 ml *E. coli* cultures grown overnight at 37°C in 2xYT + 100 μg/ml ampicillin medium (Molecular Cloning, A Laboratory Manual, second edition) by using Qiagen Tip-500 columns (Qiagen, Chatsworth, CA). Small mini-DNA preparations were grown in 2-5 ml of 2xYT + ampicillin medium overnight and purified using GFX reagents (Pharmacia Biotech). The integrity of each clone was validated by automated sequence analysis for any manipulated region of the *Tba* DNA polymerase coding region. Restriction endonucleases and T4 DNA ligase were from either Pharmacia Biotech (Milwaukee, WI) or New England Biolabs (Beverly, MA). Taq DNA polymerase was from Perkin-Elmer (Perkin-Elmer Corp., Foster City, CA 94404) with PCR reactions completed in a Perkin-Elmer thermocycler for 30 cycles with a 1 minute denaturation

step at 94°C, 1 minute annealing step at 50°C and a 0.5-1 minute extension step at 72°C. All oligonucleotide primers were manufactured and purified at Pharmacia Biotech. Electrocompetent XL1-blue cells were obtained from Stratagene (La Jolla, CA) for cloning purposes.

Expression of the *rTba* gene

For expression of the different forms of recombinant *Tba* DNA polymerase, a portion of the mini-preparation of vector DNA from the XL1-blue cloning step was transferred into heat-shock competent BL21(DE3) cells (Stratagene) and transformant cells selected on LB+ ampicillin medium. To evaluate expression of *rTbas*, 50 ml of 2xYT+ampicillin medium was inoculated with at least 10 colonies from either the transformant plate or a plate culture originating from at least 10 colonies. Following log-phase growth to O.D. 0.8-1.2 at 600 nm, cells were induced for recombinant protein production with 1 mM final IPTG for 3 hours at 37°C. The induced cultures were centrifuged at 4000 rpm in a IEC Centra-7 equipped with a fixed-angle rotor (International Equipment Company, USA) for 15 minutes at 4°C. The cell paste was immediately frozen at -20°C to preserve intact recombinant proteins until lysates were prepared.

For lysate preparation, the cell paste was resuspended at twice the cell weight in TMN buffer (50 mM Tris-HCl, pH 8.5, 10 mM magnesium chloride, 16 mM ammonium sulfate) with 500 μg/ml lysozyme and incubated for 15 minutes with occasional mixing. The resuspension

was made 0.1% with Triton X-100 and Tween 20, freeze-thawed 2 times at -80°C, then made 1 mM final with PMSF. Following microcentrifugation at top speed, the supernatant which contains the recombinant *Tba* DNA polymerase was filtered through a 0.2 micron syringe filter and stored at 4°C.

Purification of the *rTba*(exo-)

BL21(DE3) cells transformed with p22b, *rTba*(exo-) were grown to log phase in a 15 L fermentation run containing terrific broth (Molecular Cloning, A Laboratory Manual, second edition) and 100 μg/ml ampicillin. Cells were induced with 1 mM IPTG for 3 hours and the cell paste collected and frozen at -20°C. 60 g of the cell paste was diluted with 180 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 0.2 mg/ml lysozyme), sonicated, then centrifuged for 30 minutes at 10K rpm at 4°C in a Beckman centrifuge equipped with a JA-10 rotor. The supernatant containing soluble recombinant protein was heated to 80°C for 20 minutes to denature contaminating *E. coli* proteins, then cooled to 4°C. To remove the contaminating DNA, the supernatant was made 0.6% final with polyethylenimine (PEI, Sigma), stirred on ice for 30 minutes and followed by centrifugation at 10k, 30 minutes, 4°C. The supernatant containing the semi-purified *rTba*(exo-) was stored at 4°C.

To further purify the *rTba*(exo-), 160 ml of the PEI supernatant was passed onto a Q-Sepharose anion exchange

resin (Pharmacia) previously equilibrated against 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM PMSF, 1 mM DTT and the bound *rTba*(exo$^-$) eluted using a 0-1 M KCl gradient. Approximately 20 μl of the Q-Sepharose column fractions were evaluated by using either Silver (Bio Rad) or Coomassie Blue staining of the protein in SDS-PAGE under reducing conditions. A Q-Sepharose pool was collected based on visualization of the fractions containing the most *rTba*(exo$^-$) in the absence of contaminating *E. coli* proteins. This pool was dialyzed versus buffer A (50 mM Tris-HCl, pH 8.5, 20 mM ammonium sulfate, 0.1 mM EDTA, 1.0 mM DTT, 10% glycerol and 0.1% Tween 20) and passed over Heparin Sepharose CL-6B resin (Pharmacia). After washing with buffer A to return to baseline, the *rTba*(exo$^-$) was eluted with a 20-300 mM ammonium sulfate gradient in buffer A. The best fractions were evaluated as described above for the Q-Sepharose Fast Flow column and combined to yield a 1.5 L pool. The final *rTba*(exo$^-$) preparation was obtained by stir-cell concentrating the Heparin pooled fractions to approximately 120 ml and dialyzing into storage buffer (50% glycerol, 50 mM ammonium sulfate, 20 mM Tris-HCl, pH 8.5, 0.1 mM EDTA, 1 mM DTT, 0.5% NP40, 0.5% Tween 20).

Physical characterization of native *Tba* DNA polymerase and *rTba*(exo$^-$)

A semi-purified preparation of native *Tba* DNA polymerase was electrophoresed by SDS-PAGE then immunoblotted in CAPS buffer (10 mM, pH 11) to

nitrocellulose. The membrane was stained with imido black and the approximately 90 kD band corresponding to native *Tba* DNA polymerase excised from the membrane and delivered to the Protein-Nucleic Acid Shared Facility at the Medical College of Wisconsin for N-terminal sequence evaluation. The N-terminal sequence and the amino acid composition of *rTba*(exo$^-$) was determined in a similar fashion except that the material used was obtained directly from the pooled Heparin column and dialyzed into phosphate buffer (20 mM potassium phosphate, pH 7.5, 0.5 mM sodium EDTA, 50 mM potassium chloride, 1 mM DTT, 5% glycerol, 0.1% NP-40, 0.1% Tw20)

Biochemical characterization of recombinant *Tba* DNA polymerase proteins

Contaminating nickase and endonuclease levels were evaluated for purified preparations. The nickase levels are determined using phiX-174 RFI DNA as the substrate. Samples are electrophoresed on a 1% agarose gel in Tris-Acetate buffer to separate supercoiled DNA (Form I), covalently closed circular DNA (Form II) and linear DNA (Form III). The standard assay conditions are: 10 mM Tris-HCl (pH 8.3), 2 mM magnesium chloride, 50 mM potassium chloride, 0.01% gelatin and 66.6 μg/ml ϕX174 DNA in 30 μl at 65°C for 1 hour.

To detect contaminating endonuclease activity in the polymerase samples, lambda DNA is used as a substrate under the following assay conditions: 1X OPA buffer (Pharmacia Biotech), 10 mM Tris acetate, pH 7.5, 10 mM

magnesium acetate and 50 mM potassium acetate plus 20 μg/ml lambda DNA in a 50 μl reaction at 65°C for 16 hours. These digested samples are electrophoresed on 1% agarose gels to look for digestion of the lambda DNA.

The ability of DNA polymerases to incorporate dNTPs into an oligonucleotide primer reflects one activity of the enzyme. The standard M13/primer assay conditions are as follows: 25 mM glycine (pH 9.3), 50 mM potassium chloride, 2 mM magnesium chloride, 1 mM DTT, 20 μg/ml M13mp18(+) DNA, 6 μg/ml single stranded primer, 0.2 mM dTTP, dCTP and dGTP, 0.1 mM [α-^{32}P]-dATP (5 μCi/ml) and 100 μg/ml BSA in a 50 μl reaction at 70°C for 10 minutes. Several dilutions of recombinant polymerases or those in lysate preparations are typically assayed to insure that the conditions reflect the linear range of the enzyme.

For analysis of the polymerases to incorporate FL- or CY5 labeled-ddNTPs in the APEX format, different dilutions of each enzyme preparation were tested in the following format.

For oligonucleotide binding to Covalink microwell plates (Nunc, Naperville, IL), ~20 ng/rxn of primer oligonucleotide was heat treated at 100°C for 3 minutes, then placed on ice for 5 minutes. The oligonucleotide was diluted to 0.25 ng/μl with 10 mM 1-methylimidazole, pH 7.0. and 75 μl added to each well. 25 μl of 200 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was added to each well, the wells covered with 96-well tape and incubated at 50°C overnight. Following incubation, the

wells were washed 3x with immunowash (0.25% SDS or Tween 20, 0.4 N NaOH) and rinsed one time with either 1X PBS or ddH_2O. These wells are now compatible for APEX reactions taking place in an incubator format.

An individual APEX reaction typically consists of 0.25-1.0 ng/μl template, 0.5-1.0 μg thermostable DNA polymerase, 1 μM dye-ddNTP, 1 μM ddNTP mix (lacking the dye-ddNTP chosen) diluted to a total volume of 100 μl (Covalink plates) or 50 μl (Nucleolink plates) with TSP buffer (10 mM potassium chloride, 20 mM Tris-HCl, pH 8.0, 10 mM ammonium sulfate, 2 mM magnesium sulfate, 0.1% Triton X-100). (For a general description of APEX, see Shumaker, J.M., et al., Human Mutation 7:346-354, 1996.) After sealing the wells with tape, the reaction for the Covalink plates takes place in a 72°C oven for 30 minutes.

APEX reactions are evaluated using an antibody/colorimetric detection system. Following completion of the APEX reaction, the plates are washed 3-4x with 150 of 1X PBS. Open sites on the microwells are blocked with 3% BSA in 1X PBS for 30 minutes at room temperature. Anti-CY5 or anti-FL antibody (1:500 dilutions in 3% BSA solution) is next added for 60 minutes and the plates washed 3-4x with 150 μl of 1XPBS containing 0.05% Tween 20. After rinsing with 1X PBS or ddH_2O, 100 μl of *p*-nitrophenylphospate (PNPP) is added and the color allowed to develop at room temperature on the bench. Optical density is measured with an endpoint

ELISA Spectromax Pro software package at 405 nm in a Spectromax 250 microwell plate spectrophotometer (Molecular Devices).

Table 1, below, tabulates the results of experiments analyzing the APEX activity of *rTba*(exo⁻) during classical purification.

TABLE 1

DNA polymerase	APEX activity (OD @405 nm) (A)	A/wt[a]
rTba(exo⁻), lysate	0.12	0.2
rTba(exo⁻), Q-Sepharose	0.18	1.5
rTba(exo⁻), Heparin	0.42	1.6
rTba(exo⁻), Heparin, storage	0.34	1.4

[a] A_{405nm} measurements were normalized to the mass of protein used in the assay.

Results

A *Tba* DNA polymerase clone was sequenced on both strands and found to encode a potential DNA polymerase. The cloned gene contains a 1 bp deletion near the 3′ end of the molecule that would result in premature truncation and a truncated version of recombinant *Tba* DNA polymerase. This site was repaired based on the homology to Deep Vent and Pfu amino acid sequence and both the defective and repaired gene expressed via a T7 promoter system in bacteria. The recombinant *Tba* DNA polymerase from the repaired gene was expressed at high levels and at the appropriate size when compared to the native *Tba* DNA polymerase by SDS-PAGE analysis of heat-treated bacterial lysates. As predicted, the defective gene

resulted in a shorter form. Examination of the recombinant polymerases' ability to incorporate dNTPs was demonstrated either by using a nonradioactive DNA polymerase assay (Boehringer Mannheim) or an M13/primer incorporation assay (see Methods). These analyses showed that the full-length recombinant *Tba* DNA polymerase, but not the carboxy-truncated form, is an active DNA dependent DNA polymerase at high temperatures.

While the normal recombinant *Tba* DNA polymerase was able to incorporate dNTPs in the above assay formats, to date we have been unable to demonstrate that it is capable of functioning in the arrayed-primer extension format. Because early experiments showed that commercially-available Deep Vent (New England Biolabs, Beverly, MA) does not incorporate ddNTPs in APEX, but Deep Vent(exo$^-$) (New England Biolabs) does, we proposed that APEX requires polymerase activity in the absence of the 3'-5' exonuclease activity. In most chip technologies primers are in the 3'-5' orientation and are not substrates for 3'-5' exonucleases. The orientation of our primers, however, is 5'-3'. Therefore, we decided an exo$^-$ enzyme is necessary because use of exo$^-$ enzymes in normal primer extension applications and PCR can often be problematic. Thus, there may be an advantaged in using an exo$^-$ polymerase in APEX, in addition to other applications. With that in mind, a recombinant form of *Tba* DNA polymerase was engineered where the FDIET

sequence in the exonuclease domain is altered to FAIAT (termed "*rTba*(exo$^-$)").

To determine whether *rTba*(exo$^-$) exhibited a decrease in proofreading activity, an exonuclease assay was performed that is based on having a mispaired 3' end of a primer oligonucleotide when hybridized to the template strand (in this case M13 DNA). This type of assay relies on the ability of the enzyme's proofreading function to excise the mispaired 3' sequence, thereby allowing the polymerase function to incorporate radioactively labeled dNTPs. As Table 2 illustrates, all of the polymerases tested are able to effectively incorporate the labeled dATP as long as there is a perfect match between the 3' end of the primer and the template. However, only native *Tba* DNA polymerase and wild-type Deep Vent displayed the ability to excise the 3 or 6 bases of mispaired nucleotides to enable subsequent polymerization. Thus, the FDIET to FAIAT alterations significantly diminished 3'-5' exonuclease activity in the recombinant *Tba* DNA polymerase, especially when comparing the native form to the exonuclease-deficient *Tba* DNA polymerase.

TABLE 2

Elimination of proofreading exonuclease in recombinant *Tba* DNA polymerase

DNA polymerase	perfect homology (cpm incorporated)	3 bp mismatch[a] % of perfect match	6 bp mismatch[a] % of perfect match
native *Tba* DNA polymerase	14,658	75	69
rTba(exo⁻)	12,119	5	2
Deep Vent	11,587	99	70
Deep Vent(exo⁻)	8,540	9	6
AmpliTaq	15,522	9	8

[a] Primers with a mismatched 3' terminus were annealed to single-stranded M13 DNA. Polymerase activity (cpms incorporated) is possible only if exonuclease activity is present to first excise the mismatched bases. Under our conditions, extension is not possible off a mismatched primer terminus.

The ability of *rTba*(exo⁻) to function in the APEX format was tested with material purified by Q-Sepharose and Heparin chromatography (see Table 1). In the APEX format, the primer oligonucleotide is bound to a microwell support where it hybridizes with a template oligonucleotide in an orientation compatible with the ability of a polymerase to incorporate nucleotides. Unlike purified native *Tba* DNA polymerase, the *rTba*(exo⁻) is able to incorporate FL-ddATP in the primer extension format. From the relative APEX activities per weight of enzyme added (see column 4, Table 1, A/wt), it appears that *rTba*(exo⁻) is inhibited when in a lysate preparation as compared to the more purified forms (i.e. Q-Separose, Heparin). In addition, using the defined assay conditions (see Methods), it appears that purified KlenTaq with a mutation at 667 (Taquenase, Wayne M. Barnes, Washington University, School of Medicine, St.

Louis, MO 63110) performs about many times better than either Deep Vent(exo$^-$) or *rTba*(exo$^-$) in the APEX format.

Having the purified versions of both native and recombinant *Tba* DNA polymerase at hand enabled us to determine that the N-terminal sequence was intact and agreed with the sequence predicted from the cloned *Tba* DNA polymerase gene.

B. *Tba*(exo$^-$) DNA polymerase mutants

Methods: In general

The purpose of the experimentals below is to design variations of the thermostable recombinant DNA polymerase described above originating from *Thermococcus barosii* that are exonuclease deficient and have altered nucleotide substrate specificities.

As described above, the *rTba* DNA polymerase gene was made exonuclease deficient by changing the aspartate and glutamate residues in the FDIET consensus region to alanines as described above. This *rTba*(exo-) gene was then mutated in the KILANSFY (SEQ ID NO:3) (aa 487-494) consensus region.

Site-directed mutagenesis (U.S.E. mutagenesis kit, Pharmacia Biotech or Deng, et al., Anal. Biochem. 200, 81C, 1992) was performed as described above, such that the I, L, A, N, S, and F residues were changed to tyrosines (Y). Table 3, below, lists these mutations.

To effectively assess their activities in the APEX reaction, the mutants were purified from the crude heat-treated lysate. This was accomplished by cloning a His-

Tag sequence onto the C-terminus of the mutant genes and purifying by metal-chelation chromatography. The amount of His-tagged protein isolated was quantified by ELISA.

A summary of the mutants' activities in APEX reactions are described below in Table 3. Briefly, the APEX reactions were performed in a microwell system in which a primer was fixed to the solid surface via its phosphate at the 5' end. A template sequence oligo, buffer, nucleotides, enzyme and fluorescently-labeled nucleotide (deoxy-, dideoxy-, or ribonucleotide) were introduced into the well. The reactions were incubated at 72°C for 30 minutes, and the wells washed to terminate the reactions. Detection of incorporated Fl-(d,dd, or r)NTP was accomplished by incubating with anti-fluorescein Ab conjugated to alkaline phosphatase, adding *p*-nitrophenyl phospate (pNPP) substrate and reading the optical density at 405 nm.

Three isolated clones of each mutant were tested in triplicate at two enzyme concentrations to insure linearity of the assay with respect to enzyme activity. The average activity (normalized to amount of *rTba* protein in the assay) for each mutant is shown in Table 3.

Conclusions

Activities of the I, L, A, N, and F tyrosine mutants can be compared to that of *rTba*(exo-)/His DNA polymerase. The ability of each of the mutants to incorporate deoxynucleotides is not significantly altered by the

mutants except for the N491Y mutant in which this activity is decreased 3-4-fold. All mutants were assayed for their ability to incorporate a dideoxy terminator and ribonucleotides. Of interest is the dideoxy activity of the L489Y/His mutant which is approximately 3-fold greater than that of the exo-/His enzyme. This mutant also displays enhanced ability to incorporate ribonucleotides as well.

It is evident that the perturbation at the L489 residue has an effect on nucleotide substrate specificity and is a key residue involved in this function.

TABLE 3

KILANSFY "Tyrosine" Mutant Activities

	Deoxy Activity/ng (x 1000)	Dideoxy Activity/ng (x 1000)	Ribo Activity/ng (x 1000)	Dideoxy: Deoxy	Ribo: Deoxy
Exo-/His	13.0	1.2	0.4	0.09	0.03
I488Y/His	14.1	0.2	0.1	0.01	0.01
L489Y/His	10.5	3.5	2.1	0.33	0.20
A490Y/His	13.6	0.6	0.2	0.04	0.01
N491Y/His	3.5	0.1	0.0	0.03	---
F493Y/His	16.6	1.7	0.6	0.10	0.04

CLAIMS

We claim:

1. A *Thermococcus barosii* DNA polymerase, wherein the polymerase has a reduced 3'-5' exonuclease activity.

2. The polymerase of claim 1, wherein the 3'-5' exonuclease activity is reduced at least 50% as measured by the ability of the polymerase to incorporate dNTPs into an oligonucleotide primer with mismatches.

3. The polymerase of claim 2 wherein the reduction is at least 75%.

4. The polymerase of claim 3 wherein the reduction is 90%.

5. The polymerase of claim 1 wherein the FDIET amino acid sequence has been altered to FAIAT.

6. A method of DNA polymerization, comprising exposing the polymerase of claim 1 to a DNA template, primer, dNTPs, and other reagents necessary for polymerization.

7. A method of arrayed primer extension, comprising the steps of

a. exposing the polymerase of claim 1 to bound oligonucleotide primers that are arrayed on a solid support, chain terminators, and to other reagents necessary for primer extension, and

b. incorporating chain terminators, whereby primer extension ceases.

8. The polymerase of claim 1 wherein amino acid residue 489 has been altered.

9. The polymerase of claim 8 wherein amino acid residue 489 is selected from the group consisting of tyrosine and phenylalanine.

10. The polymerase of claim 9 wherein residue 489 is a tyrosine.

11. The polymerase of claim 8 wherein the polymerase has an increased ability to incorporate dideoxy nucleotides compared to *Tba* DNA polymerase with no alteration at residue 489.

12. The polymerase of claim 11 wherein the increase is at least 2-fold.

13. The polymerase of claim 12 wherein the increase is at least 3-fold.

14. The polymerase of claim 8 wherein the polymerase has an increased ability to incorporate ribonucleotides compared to *Thermococcus barosii* DNA polymerase with no substitution at residue 489.

15. The polymerase of claim 14 wherein the increase is at least 2-fold.

16. The polymerase of claim 15 wherein the increase is at least 3-fold.

17. The polymerase of claim 16 wherein the increase is at least 5-fold.

18. A method of DNA polymerization, comprising exposing the polymerase of claim 8 to a DNA template, primer, dNTPs, and other reagents necessary for polymerization.

19. A method of arrayed primer extension, comprising the steps of

a. exposing the polymerase of claim 8 to bound oligonucleotide primers that are arrayed on a solid support, chain terminators, and to other reagents necessary for primer extension, and

b. incorporating chain terminators, whereby primer extension ceases.

INTERNATIONAL SEARCH REPORT

Intern. Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/12 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 22389 A (PHARMACIA BIOTECH INC) 25 July 1996 see the whole document ---	1-7
A	WO 97 09451 A (CHATTERJEE DEB K ;HUGHES A JOHN JR (US)) 13 March 1997 see the whole document ---	1-7
A	US 5 489 523 A (MATHUR ERIC J) 6 February 1996 cited in the application see the whole document ---	1-7

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	SOUTHWORTH M W ET AL: "CLONING OF THERMOSTABLE DNA POLYMERASES FROM HYPERTHERMOPHILIC MARINE ARCHEA WITH EMPHASIS ON THERMOCOCCUS SP. 9 N-7 AND MUTATIONS AFFECTING 3'-5' EXONUCLEASE ACTIVITY" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, no. 11, 28 May 1996, pages 5281-5285, XP000652319 see table 1 ---	1-7
A	BERNAD A ET AL: "A CONSERVED 3'->5' EXONUCLEASE ACTIVE SITE IN PROKARYOTIC AND EUKARYOTIC DNA POLYMERASES" CELL, vol. 59, 6 October 1989, pages 219-228, XP002019904 cited in the application see the whole document ---	1-7
A	WO 92 10587 A (AFFYMAX TECH NV) 25 June 1992 see the whole document ---	7,19
A	OROSKAR ET AL: "DETECTION OF IMMOBILIZED AMPLICONS BY ELISA-LIKE TECHNIQUES" CLINICAL CHEMISTRY, XP002074418 see the whole document -----	7,19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/16170

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